

# A Triple Quadrupole LC/MS/MS Method for Quantitative Analysis of Methylenedioxypropylamphetamine (MDPV) and Mephedrone, Common Components of “Bath Salts” in Urine

## Application Note

Forensic Toxicology

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### Abstract

Due to the emerging use of synthetic cathinones – compounds widely marketed as “Bath Salts” – today’s forensic laboratories are challenged to screen, confirm, and quantify the controlled forms of those compounds in biological matrices with confidence. This application note describes and evaluates a robust quantitative method for the analysis of two controlled synthetic cathinones, 3, 4-methylenedioxypropylamphetamine (MDPV) and 4-methylmethcathione (mephedrone), in urine. The method is shown to demonstrate excellent linearity, lower limit of detection (LOD), reproducibility/precision and lower limit of quantitation (LOQ), with no interferences from structurally similar compounds, and with negligible carryover.



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## Introduction

Synthetic cathinones, such as 3,4-methylenedioxypropylrovalerone (MDPV) and 4-methylmethcathinone (mephedrone), are central nervous system (CNS) stimulants, similar in action to methamphetamine and Ecstasy (MDMA). Figure 1 shows they are chemically akin in structure to cathinone, an active alkaloid found in the Khat plant of eastern Africa.

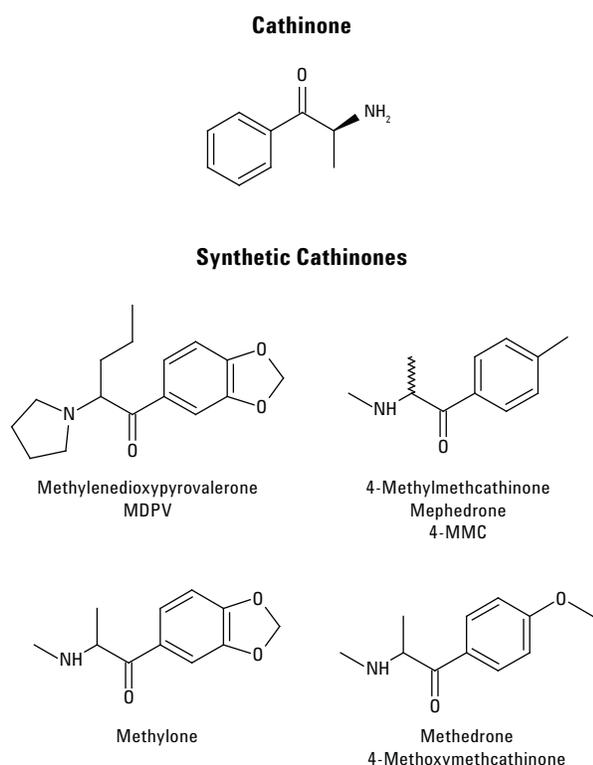


Figure 1. Cathinone and common synthetic cathinones.

In the US, synthetic cathinones are marketed as “Bath Salts” under a variety of brand names. In particular, MDPV and mephedrone use lead to effects similar to that of methamphetamine, cocaine and Ecstasy.

The hydrochloride salt of MDPV is a white to brown powder, where Mephedrone is a white crystal or powder that can be formulated as a tablet.

The EU ruled the two drugs illegal in December 2010, where it is now illegal to use, possess, sell or manufacture MDPV and mephedrone in the US as well.

Due to these trends, forensic analysis of MDPV and mephedrone, as well as other synthetic cathinones, is expected to increase. Because these compounds are not detected through existing amphetamine screening immunoassays or confirmatory gas chromatography/mass spectrometry (GC/MS) assays, new methodology is needed. Thus, the objective of this application note is to describe a liquid chromatography-triple quadrupole mass spectrometry (LC/MS/MS) method for screening, confirmation, and quantification of MDPV and mephedrone in urine. Developed by Sterling Reference Laboratories and Agilent Technologies, as of the date this application note was published, the analytical method has been used to effectively analyze 561 urine samples. The overall positivity rate was 8% (45 specimens), of which 41 (7.3%) were positive for MDPV, three (0.5%) were positive for mephedrone, and one (0.2%) was positive for both MDPV and mephedrone.

## Experimental

### Method Overview

Spiked synthetic urine samples were prepared and then extracted using cation exchange solid phase extraction (SPE) columns. SPE was used rather than simple sample dilution and direct injection ("dilute and shoot") because the SPE method introduces much cleaner samples into the mass spectrometer. As a result, ion suppression and ion source cleaning tasks are minimized, and sensitivity is enhanced. Dilute and shoot methods introduce dirtier samples – raw diluted urine – into the mass spectrometer. However, forensic laboratories performing only a small number of bath salts analyses and that do not mind frequent source cleaning may prefer to "dilute and shoot."

The extracted samples were injected into a LC/MS/MS system equipped with electrospray ionization. Two multiple reaction monitoring (MRM) transitions were monitored for each analyte and internal standard. Retention time and the ratios of the selected ions, relative to the internal standards, were used for detection and quantification. Calibration curve development and quantitative analysis were performed using MassHunter data analysis software. Quantitative analysis was performed through interpolation of the analyte response against the calibration curves. The analytical method was evaluated based on the following criteria: linearity, lower LOD and LOQ, reproducibility, interferences, and carryover.

### Synthetic Urine, Calibrator, Quality Control, and Internal Standard Solutions

Synthetic urine (solution to be spiked and the negative control or blank) was prepared by dissolving the following in 800 mL deionized water. Deionized water was then added to bring the final volume to 4,000 mL.

20.0 g	NaCl (ACS reagent grade)
2.0 g	creatinine (Sigma Cat. No. C4255-100G)
40.0 g	urea (ACS reagent grade)
38.6 g	monosodium phosphate monohydrate (ACS reagent grade)
32.3 g	disodium phosphate heptahydrate (ACS reagent grade)
4.0 g	sodium azide (ACS reagent grade)
3 to 5 drops	yellow food coloring (food grade, McCormick)

The pH of the synthetic urine was then adjusted to 6.5 using 1% HCl.

**Calibrator solutions** were made with MDPV (Cayman, Cat. No.10684) and mephedrone (Cerilliant Cat. No. M-138) stock solutions dissolved in methanol. To construct calibration

curves for MDPV and mephedrone over the range of 1 to 5000 ng/mL, calibrator compound was spiked in synthetic urine at the 1, 5, 10, 25, 50, 100, 500, 1,000, and 5,000 ng/mL level.

**Quality Control (QC) specimens.** Three QC specimens were prepared in synthetic urine: negative, 40% of positive cutoff and +25% of positive cutoff. The positive cutoff was administratively set at 25 ng/mL, thus the corresponding 40% and +25% QC specimens were nominally set at 10 ng/mL and 31 ng/mL, respectively.

**Deuterium-labeled internal standard solutions** of MDPV-D8 (Cayman, Cat. No. 10679) and mephedrone-D3 (Cerilliant, Cat. No. M-139) in HPLC-grade methanol were prepared at 500 ng/mL in deionized water.

### Sample Preparation

Figure 2 shows an overview of the cation exchange SPE sample preparation procedure. The procedure is designed to enable preparation of over 300 samples per 8-hour shift with ease.

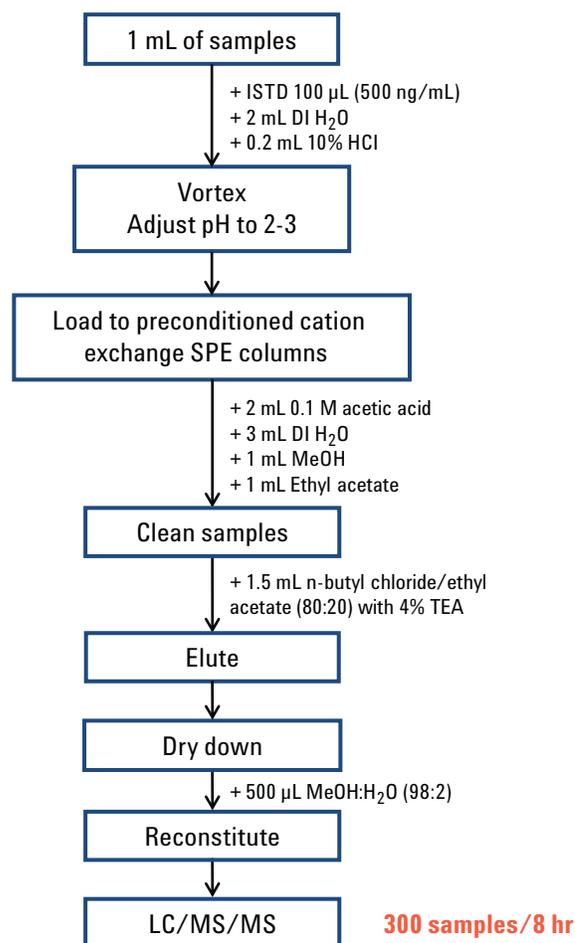


Figure 2. Sample preparation overview.

Using a calibrated micropipette, 1 mL of the calibrator, 40% QC, +25% QC, and negative control urine solutions were transferred to individual 16×100 mm labeled culture tubes. Next, using a calibrated repeating pipettor with calibrated pipette dispensers, 100 µL of the 500 ng/mL internal standard solution was transferred to the samples in the culture tubes. Deionized water (2 mL) was likewise added to each of the culture tubes. The pH was adjusted to 2–3, with the addition of 0.2 mL of 10% HCl to each of the culture tubes.

Extraction was performed on a Multi-prep SPE workstation (Biochemical Diagnostics). The cation exchange SPE columns (Biochemical Diagnostics, Cat. No. 1410082-0, GV-65), were conditioned with 1 mL of methanol followed by 1 mL of 5% sodium bisulfite which were allowed to flow through the system by gravity.

To extract the prepared samples, each was poured into the corresponding labeled SPE column. Next 2 mL of 0.1 M acetic acid was pipetted into each SPE column, followed by 3 mL of deionized water, 1 mL of methanol and finally, 1 mL of ethyl acetate. Between each solution addition, the liquid was allowed to flow by gravity until there was no liquid observed above the column bed.

Sample elution was performed outside of the vacuum box into elution tubes. Elution solvent (1.5 mL), n-butyl chloride/ethyl acetate 80:20 with 4% TEA, was pipetted into each column and allowed to flow by gravity until there was no liquid above the column bed. The elution tubes were placed in aluminum dry down blocks and evaporated to dryness under a gentle stream of nitrogen at 34–40 °C.

The samples were reconstituted with 0.5 mL of methanol:deionized water (2:98) and let to sit for 20 minutes at ambient temperature before transferring to autosampler vials. Once the samples were reconstituted, they were ready for LC/MS/MS analysis.

## LC/MS/MS Analyses

LC/MS/MS analyses were performed using an Agilent 1200 Series LC System coupled to an Agilent 6460 Series Triple Quadrupole Mass Spectrometer. The total run time is 4.2 minutes per sample. The LC System was equipped with an autosampler, degasser, binary pump, and thermostated column compartment. Separation was performed on an Agilent Polaris C18 column. The LC operating parameters are shown in Table 1.

Table 1. LC Operating Parameters

Column	Agilent Polaris C18, 50 × 2.0 mm, 5 µm			
Injection volume	1 µL			
LC gradient	Time	Flow	Mobile Phase B	Mobile Phase A
	0	0.8	2%	98%
	2	0.8	30%	70%
	2.5	0.8	90%	10%
	3.5	0.8	90%	10%
	3.6	0.8	2%	98%

Mobile phase A: 100% deionized water containing 0.1% formic acid

Mobile phase B: 100% methanol containing 0.1% formic acid

The Agilent 6460 Series Triple Quadrupole LC/MS/MS System was equipped with an electrospray ionization (ESI) source operated in positive ion mode. The MS operating parameters are shown in Table 2.

Table 2. MS Operating Parameters

Nebulizing gas	Nitrogen, (ultra high purity), 99.999%	
Collision cell gas	Nitrogen, (ultra high purity), 99.999%	
Ion source parameters	Gas temperature	300°C
	Gas flow	10 L/min
	Nebulizer	20 psi
	Sheath gas heater	350°C
	Sheath gas flow	8 L/min
	Capillary	4,000 V
Detector parameters	Nozzle voltage	0 V
	EMV	200

Two MRM transitions were monitored for each analyte and internal standard. The MRM transitions and MS/MS specific parameters for the mephedrone and MDPV compounds monitored are shown in Table 3.

Table 3. MRM Transitions and MS/MS Parameters

Compound	Precursor ion	Product ions monitored	Dwell time (ms)	Fragmentation voltage	Collision energy voltage
Mephedrone	178.1	160.1/145.0	50	95	8/20
Mephedrone-D3	181.1	163.1/148.1	50	90	8/20
MDPV	276.2	135.0/126.1	50	130	24/24
MDPV-D8	284.2	134.5/149.0	50	130	28/32

## Calibration Curve Construction

In order to construct calibration curves for MDPV and mephedrone over the range of 1 to 5 000 ng/mL, five replicates (one injection of five extractions, n = 5) were made at each level (1, 5, 10, 25, 50, 100, 500, 1,000, and 5,000 ng/mL). Calibration curves were constructed by Agilent MassHunter Software using least-squares linear regression of the ratio of the quantitation ion abundance of the analyte/internal standard versus the concentration of the calibrators.

## Results and Discussion

The total ion chromatogram (TIC) and MRM chromatograms for the MDPV and mephedrone, deuterated and nondeuterated forms in synthetic urine at 25 ng/mL, are shown in Figure 3. Due to the selectivity of the LC/MS/MS technique, chemical noise was negligible and response was strong. Ideally-shaped Gaussian peaks for quantitation were also observed.

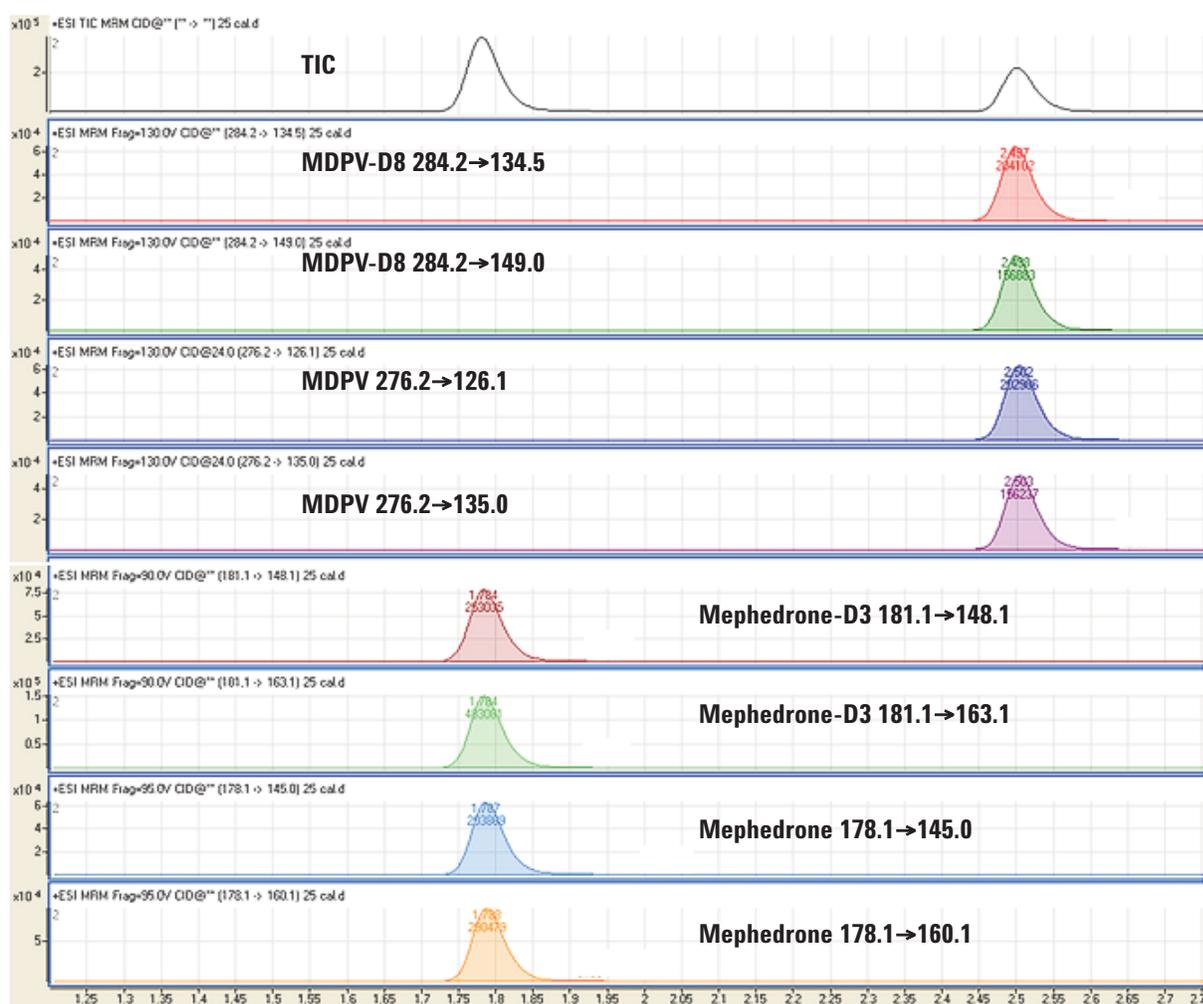


Figure 3. Total ion chromatogram (TIC) and MRM chromatograms for the MDVP and mephedrone deuterated and non-deuterated forms, in synthetic urine. Response for all compounds at 25 ng/mL was strong.

Calibration curves for MDPV and mephedrone spiked in synthetic urine over the range of 1 to 5,000 ng/mL are shown in Figure 4. Method linearity was excellent over the entire range of concentrations, including at the low end of the calibration curve, with an average correlation coefficient ( $R^2$ ) greater than 0.999.

Based on the five replicates, an average signal-to-noise ratio (S/N) of  $19 \pm 6$  and  $24 \pm 9$  was obtained at 1 ng/mL for MDPV and mephedrone, respectively. Theoretically, a lower limit of detection (LOD) could be reached, but 1 ng/mL was determined to be practical for most routine analyses. Figures 5 and 6 show the method response for MDPV and mephedrone, respectively, at 1 ng/mL.

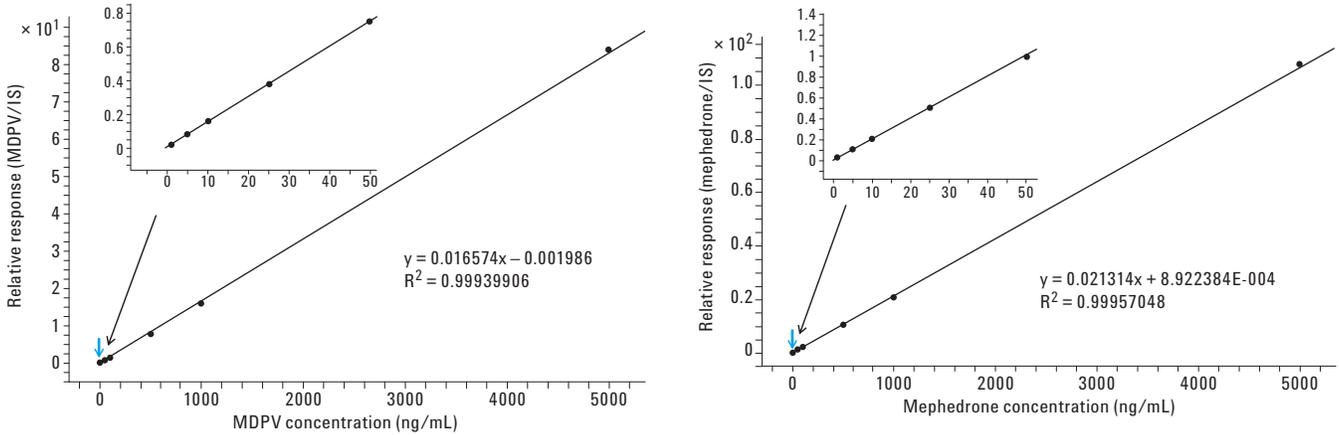


Figure 4. MDPV and mephedrone calibration curves demonstrated the excellent linearity of the method, even at low analyte concentration (insets).

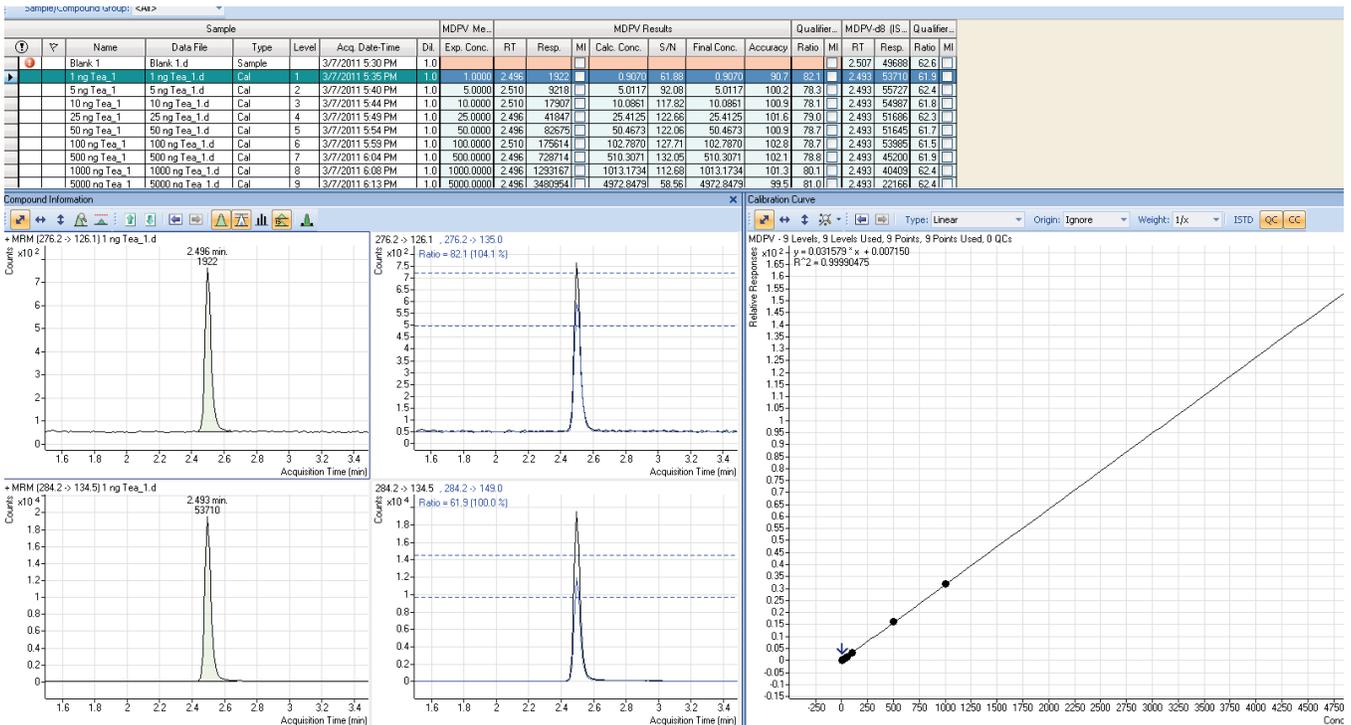


Figure 5. Method response for MDPV at 1 ng/mL, the LOD.

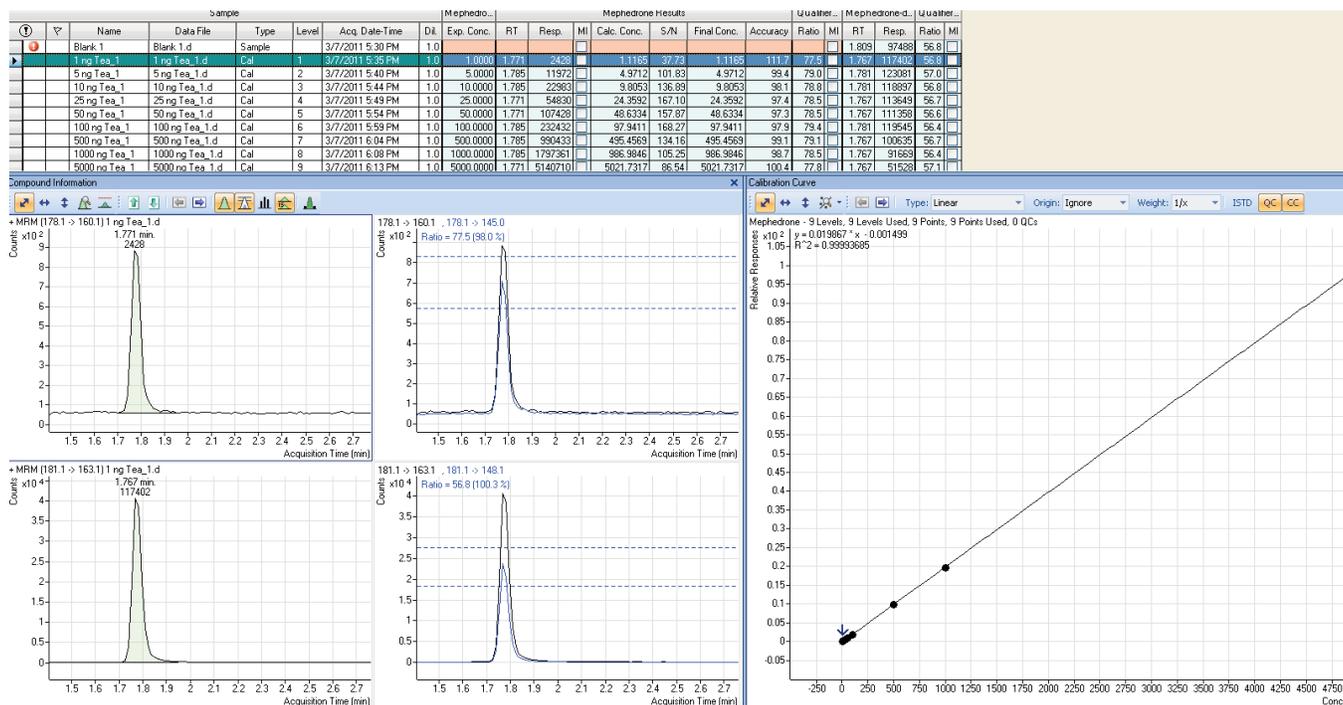


Figure 6. Method response for mephedrone at 1 ng/mL, the LOD.

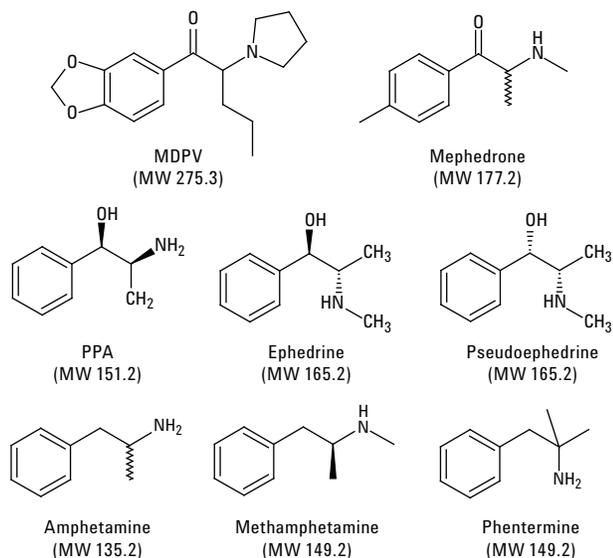
The excellent reproducibility (precision at  $n = 5$ ) of the method for the nine calibrators spiked in synthetic urine is shown in Table 4. Imprecision was within 5% relative standard deviation (RSD). At 5 ng/mL, the level of the second to the lowest calibrator, at least 80% accuracy was obtained when developing the calibration curves. Thus, 5 ng/mL was chosen as the reasonable limit of quantitation (LOQ) for the analytical method.

Table 4. Method Precision ( $n=5$ ) for the Calibrator Compounds in Synthetic Urine

Expected conc ng/mL	Average conc. ng/mL	
	MDPV (RSD%)	Mephedrone (RSD%)
1	1.33 (3.83%)	1.21 (4.91%)
5	4.98 (3.98%)	4.96 (3.14%)
10	9.53 (2.49%)	9.69 (1.72%)
25	22.61 (1.84%)	23.94 (1.81%)
50	45.65 (2.64%)	47.17 (0.82%)
100	93.81 (3.12%)	96.81 (1.32%)
500	479.46 (0.92%)	485.71 (1.79%)
1000	991.13 (1.40%)	975.75 (0.98%)
5000	5042.34 (0.33%)	5045.73 (0.39%)

Though no cut-off values for MDPV and mephedrone have been nominated or established, 25 ng/mL seems a suitable choice based on the results described here.

To test for possible interferences, six compounds similar in structure to MDPV and mephedrone were spiked in the blank urine to reach the relatively high concentrations of  $1 \times 10^6$ ,  $1 \times 10^6$ ,  $1 \times 10^6$ ,  $5 \times 10^4$ ,  $5 \times 10^3$ , and  $5 \times 10^3$  ng/mL for phenylpropranolamine (PPA), ephedrine, pseudoephedrine, phentermine, amphetamine and methamphetamine, respectively. Figure 7 shows the structures and molecular weights of these compounds. Because their molecular weights are different from both MDPV and mephedrone, no interferences were expected to be observed. The samples were prepared, extracted and run through the LC/MS/MS system as described earlier.



As expected, no interferences were found. Figure 8 shows the MRM chromatograms for the blank urine sample spiked with ephedrine at  $1 \times 10^6$  ng/mL, a high concentration in comparison to that expected for the target synthetic cathinones. The four peaks shown are of the MDPV-D8 and mephedrone-D3 transitions.

To check for carryover, the negative control urine was analyzed after five injections of 10,000 ng/mL MDPV and mephedrone. Small peaks resulting from carryover were observed. Figure 9 and Figure 10 show the calculated concentration of MDPV and mephedrone in the blank sample was 3.18 ng/mL and 1.53 ng/mL, respectively. Because both values are well below the chosen cutoff, 25 ng/mL, carryover was considered negligible.

Figure 7. Six compounds similar in structure to MDPV and mephedrone.

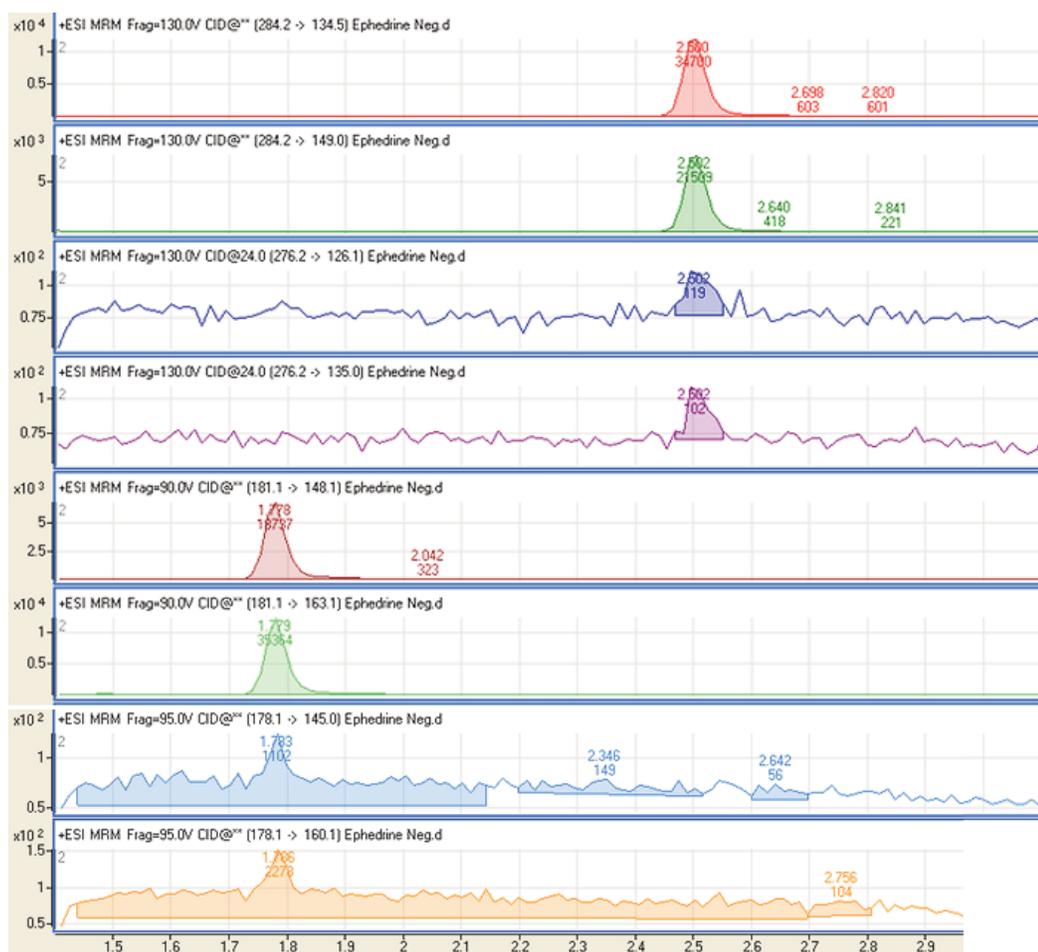


Figure 8. MRM chromatograms of blank urine spiked with ephedrine at  $1 \times 10^6$  ng/mL. No interferences were observed.

Sample										MDPV Results										Qualifier...			MDPV-d8 (S...)			Qualifier...		
①	Name	Data File	Type	Level	Acq. Date-Time	Dil.	Exp. Conc.	RT	Resp.	MI	Calc. Conc.	S/N	Final Conc.	Accuracy	Ratio	MI	RT	Resp.	Ratio	MI	RT	Resp.	Ratio	MI				
	Amp 50 cal	Amp 50 cal.d	Sample		3/7/2011 7:16 PM	1.0		2.510	80142		49.5386	130.88	49.5386		77.3		2.507	50996	62.4									
	Methamp 50 cal	Methamp 50 cal.d	Sample		3/7/2011 7:21 PM	1.0		2.510	79999		49.8823	128.19	49.8823		78.3		2.507	50956	61.8									
	Blank c	Blank c.d	Sample		3/7/2011 7:25 PM	1.0		2.510	79		0.0000	0.41	0.0000		31.4		2.507	49911	61.4									
	10000 ng1	10000 ng1.d	Sample		3/7/2011 7:30 PM	1.0		2.496	4885892		9778.8549	53.59	9778.8549		81.6		2.493	15822	62.1									
	10000 ng2	10000 ng2.d	Sample		3/7/2011 7:35 PM	1.0		2.496	4876489		9793.0743	47.02	9793.0743		82.1		2.493	15768	62.0									
	10000 ng3	10000 ng3.d	Sample		3/7/2011 7:40 PM	1.0		2.496	4861708		9740.9553	46.59	9740.9559		82.0		2.493	15805	61.7									
	10000 ng4	10000 ng4.d	Sample		3/7/2011 7:45 PM	1.0		2.496	4864968		9733.2020	47.56	9733.2020		81.7		2.479	15923	62.3									
	10000 ng5	10000 ng5.d	Sample		3/7/2011 7:49 PM	1.0		2.496	4867449		9807.4730	52.36	9807.4730		81.6		2.493	15557	61.6									
	Blank d	Blank d.d	Sample		3/7/2011 7:54 PM	1.0		2.510	5876		3.1874	3.09	3.1874		77.2		2.507	54907	61.2									
	10000 ng6	10000 ng6.d	Sample		3/7/2011 7:59 PM	1.0		2.496	4747596		9693.8852	42.33	9693.8852		82.2		2.479	15909	61.3									
	10000 ng7	10000 ng7.d	Sample		3/7/2011 8:04 PM	1.0		2.496	4781948		9947.0821	47.69	9947.0821		81.7		2.493	15223	62.1									
	10000 ng8	10000 ng8.d	Sample		3/7/2011 8:09 PM	1.0		2.496	4715491		9918.7051	41.60	9918.7051		81.3		2.479	15055	63.1									
	10000 ng9	10000 ng9.d	Sample		3/7/2011 8:14 PM	1.0		2.496	4737835		10076.8067	49.48	10076.8067		81.1		2.493	14889	63.5									
	10000 ng10	10000 ng10.d	Sample		3/7/2011 8:19 PM	1.0		2.496	4686039		9767.7849	41.79	9767.7849		82.1		2.479	15153	61.8									
	25 cal	25 cal.d	Sample		3/7/2011 8:23 PM	1.0		2.510	48042		23.1068	24.39	23.1068		79.6		2.493	51864	62.3									
	Blank e	Blank e.d	Sample		3/7/2011 8:28 PM	1.0		2.510	3914		2.1659	3.69	2.1659		83.5		2.493	51814	62.7									

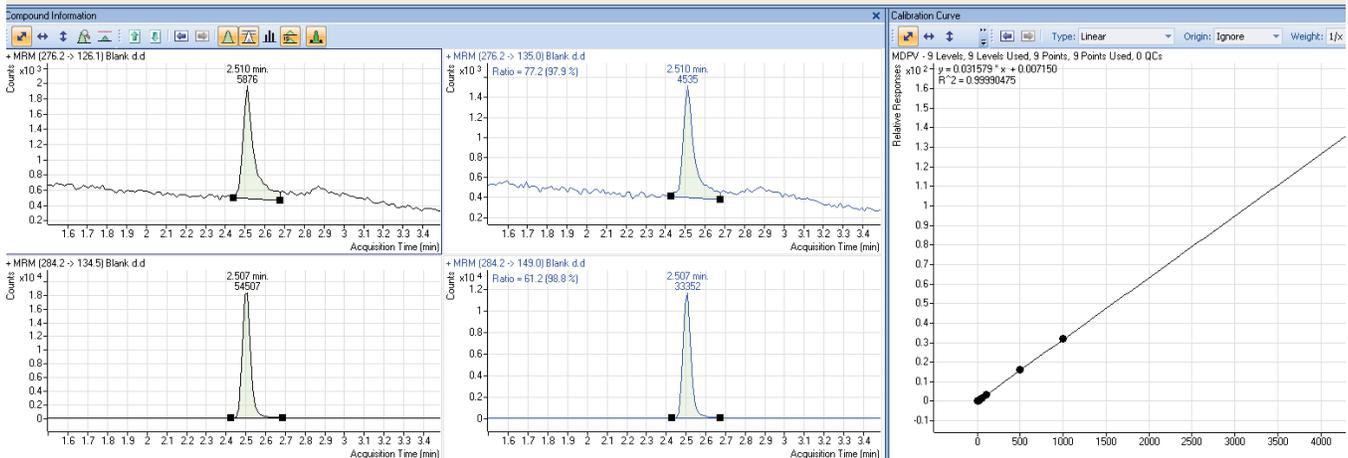


Figure 9. MRPV carryover is negligible at 3.18 ng/mL and below the cutoff value of 25 ng/mL.

Sample										Mephedrone Results										Qualifier (1781 > 14)			Mephedrone-d...			Qualifier...		
①	Name	Data File	Type	Level	Acq. Date-Time	Dil.	Exp. Conc.	RT	Resp.	MI	Calc. Conc.	S/N	Final Conc.	Accuracy	Ratio	MI	RT	Resp.	Ratio	MI	RT	Resp.	Ratio	MI				
	Amp 50 cal	Amp 50 cal.d	Sample		3/7/2011 7:16 PM	1.0		1.785	87490		46.1505	168.48	46.1505		79.1		1.781	95578	56.0									
	Methamp 50 cal	Methamp 50 cal.d	Sample		3/7/2011 7:21 PM	1.0		1.785	98872		46.7823	178.86	46.7823		78.7		1.781	106551	56.3									
	Blank c	Blank c.d	Sample		3/7/2011 7:25 PM	1.0		1.785	61		0.1030	0.71	0.1030		45.2		1.781	110646	56.0									
	10000 ng1	10000 ng1.d	Sample		3/7/2011 7:30 PM	1.0		1.771	7796121		9897.3254	61.44	9897.3254		77.7		1.767	39252	56.7									
	10000 ng2	10000 ng2.d	Sample		3/7/2011 7:35 PM	1.0		1.771	7771071		10091.3608	90.28	10091.3602		77.6		1.767	38993	56.6									
	10000 ng3	10000 ng3.d	Sample		3/7/2011 7:40 PM	1.0		1.771	7831025		10204.9412	90.71	10204.9412		76.7		1.767	38626	56.9									
	10000 ng4	10000 ng4.d	Sample		3/7/2011 7:45 PM	1.0		1.771	7831111		10085.1091	88.50	10085.1091		77.5		1.767	39085	55.7									
	10000 ng5	10000 ng5.d	Sample		3/7/2011 7:49 PM	1.0		1.771	7809960		10162.9453	88.44	10162.9453		77.7		1.767	38681	56.5									
	Blank d	Blank d.d	Sample		3/7/2011 7:54 PM	1.0		1.785	3737		1.5333	1.28	1.5333		77.6		1.781	129018	56.2									
	10000 ng6	10000 ng6.d	Sample		3/7/2011 7:59 PM	1.0		1.771	7631533		10044.2015	61.44	10044.2015		76.1		1.767	38244	56.6									
	10000 ng7	10000 ng7.d	Sample		3/7/2011 8:04 PM	1.0		1.771	7693330		10188.8224	89.21	10188.8224		75.9		1.767	38007	57.4									
	10000 ng8	10000 ng8.d	Sample		3/7/2011 8:09 PM	1.0		1.771	7575220		10139.0427	60.07	10139.0427		74.6		1.767	37610	58.0									
	10000 ng9	10000 ng9.d	Sample		3/7/2011 8:14 PM	1.0		1.771	7693415		10169.9458	88.03	10169.9433		75.3		1.767	37567	56.8									
	10000 ng10	10000 ng10.d	Sample		3/7/2011 8:19 PM	1.0		1.771	7491836		10029.6838	58.49	10029.6838		75.7		1.767	37938	56.4									
	25 cal	25 cal.d	Sample		3/7/2011 8:23 PM	1.0		1.785	68300		28.1966	17.18	28.1966		76.5		1.781	122394	56.8									
	Blank e	Blank e.d	Sample		3/7/2011 8:28 PM	1.0		1.785	4160		1.7812	2.35	1.7812		83.2		1.781	122742	56.5									

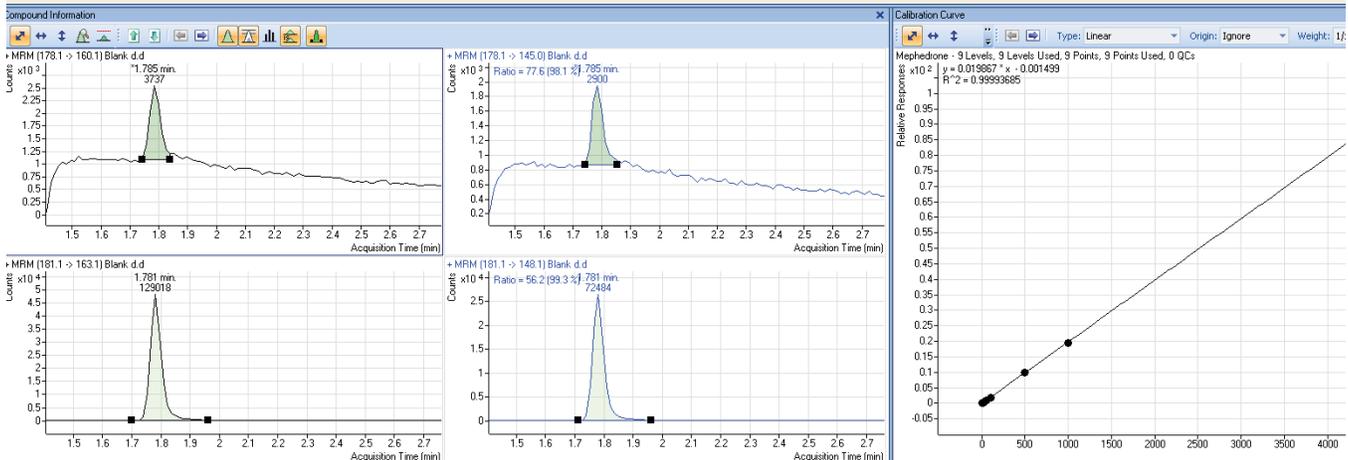


Figure 10. Mephedrone carryover is negligible at 1.53 ng/mL and below the cutoff value of 25 ng/mL.

## Conclusion

Due to its selectivity and sensitivity, LC/MS/MS is a powerful technique for screening, confirmation and quantification of synthetic cathinones, such as MDVP and mephedrone, in complex biological matrices such as urine. The LC/MS/MS analytical method described here offers forensics laboratories an easy and robust approach that demonstrates excellent linearity, LOD, reproducibility and LOQ, with no interferences from structurally similar compounds, and with negligible carryover. The analytical method can also be easily modified to include the analysis of other synthetic cathinones as the need arises.

## Further Reading

1. American Association of Poison Control Centers.  
<http://www.aapcc.org>.
2. DEA Drug Fact Sheet, Bath Salts or Designer Cathinones (Synthetic Stimulants).  
[http://www.justice.gov/dea/pubs/abuse/drug\\_data\\_sheets/Bath\\_Salts.pdf](http://www.justice.gov/dea/pubs/abuse/drug_data_sheets/Bath_Salts.pdf).
3. Federal Register Vol. 76, No. 174. September 8, 2011.
4. Spiller, H.A. et al. Clinical experience with and analytical confirmation of "bath salts" and "legal highs" (synthetic cathinones) in the United States. Clin. Toxicol. 2011; Jul;49(6): 499-505.

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